

The University of Chicago

THE EFFECTS OF CARBON DIOXIDE,
THE HYDROGEN ION, CALCIUM, AND EX-
PERIMENTAL CONDITIONING ON RE-
CONSTITUTION IN *EUPLANARIA*
DOROTOCEPHALA

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL
SCIENCES IN CANDIDACY FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOÖLOGY

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OLIN RULON

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Reprinted from
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THE EFFECTS OF CARBON DIOXIDE, THE HYDROGEN ION, CALCIUM, AND EXPERIMENTAL CONDITIONING ON RECONSTITUTION IN EUPLANARIA DOROTOCEPHALA.¹

(Six figures)

OLIN RULON

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THE effects of acids on physiological processes have long been of interest to investigators. One has but to look at the tremendous amount of literature that has accumulated on the subject to realize that the H-ion is of prime importance. It is no doubt true that many biological phenomena have been attributed directly to the external H-ion when in reality the relationship may be very indirect. Alterations of the H-ion concentration may cause an increase or a decrease in the solubility of other substances in the solution, and this factor alone may be responsible for many phenomena that have been stated as being results of the direct action of the H-ion.

The effects of external CO₂ have often been confused with the effects of the external H-ion. The alteration of physiological processes of organisms in natural waters to which acid has been added has often been stated as being the result of H-ion rather than of the CO₂ which may have been liberated on acidifying the water.

In general it has been found that increased H-ion and CO₂ concentration in the external medium serve as inhibitors to biological processes. In recent years much work has been done in altering reconstitucional and embryological development through the use of various inhibiting agents applied externally. It has been found, in general, that agents which tend to inhibit development in organisms do so differentially. In other words, the more active regions may be inhibited to a greater extent than the less active regions. Certain phenomena of twinning, exogastrulation, and various other teratological processes are evidently examples of differential inhibition.

¹ The author wishes to thank Professor C. M. Child for much valuable advice and criticism received throughout the progress of this work.

In this investigation we are concerned chiefly with the effects of the H-ion and CO₂ on reconstitucional development.

THE PROBLEM

Child (1911a) pointed out that transverse pieces of *Euplanaria dorocephala*, of a limited size and from the more posterior levels of the first zooid, reconstituted in a large number of cases into headless individuals or individuals which showed various degrees of head inhibition. The frequency of inhibited head-forms and the degree of inhibition were found to be related to the level from which the piece was taken and to its length. Short pieces and pieces from the posterior levels of the anterior zooid showed the greatest inhibition in head-form. Child has placed the reconstituted head-forms in the following classes, corresponding to the degree of inhibition:

Normal.—Head triangular with two symmetrically placed eyespots and with auricles at lateral margins.

Teratophthalmic.—Head normal in shape and with auricles in normal position, but with eyespots showing all degrees of approximation to the median plane from two distinct eyespots with pigment connected to complete cyclopia.

Teratomorphic.—Head more or less rounded in outline with single or apparently single median eye; auricles more or less anterior and showing all degrees of approximation to the median plane up to a single median auricle.

Anophthalmic.—Heads rudimentary without eyespots and with or without a single median auricle.

Acephalic.—Head completely absent.²

It subsequently has been found and sustained by Child and others that the inhibition of normal head formation in transverse pieces of *Euplanaria* is related to some inhibitory influence which arises from the posterior cut surface of the isolated piece. This factor inhibiting head development is most effective during the first 24 hours after section; yet, it may still be evidenced, as shown by delay of anterior

² Since Child (1911a, 1911b, 1911c, 1912, 1914a, 1914b, 1916, 1920, 1924), Child and Watanabe (1935), and others (Behre, 1918; Buchanan, 1922; Hinrichs, 1924) have repeatedly described and figured the different head types in *Euplanaria*, it does not seem necessary to present either drawings or photographs of them here.

section, after 3 or 4 days (Child and Watanabe, 1935). It is quite evident that this inhibiting action of the posterior cut surface is not entirely the result of stimulation by section, since this stimulation, as indicated by increased respiration and susceptibility, disappears within a few hours (Child, 1914*a*; Robbins and Child, 1920; Hyman, 1923). It is probable that the cellular activity associated with the early stages of tail development also plays a part in the inhibition. The inhibiting factor is apparently largely or wholly nervous in character and can be decreased or obliterated by delay of posterior or anterior section or by section under anesthesia (Child, 1914*b*; Buchanan, 1922; Child and Watanabe, 1935; Watanabe, 1935*b*).

In dealing with the various types of heads that arise from lots of reconstituted worms, the term "head frequency" has been used. By head frequency is meant the frequency of the various forms that reconstitute from lots consisting of pieces of a certain length and from a certain body-level of animals of the same length. If a certain lot of pieces gives a preponderance of normal and near-normal animals, we speak of the lot as having a high head frequency. If a lot gives a majority of headless or strongly inhibited head-forms, we speak of it as having a low head frequency.

Many workers have found that pieces of planarians, which would normally give a low head frequency, gave an increased head frequency when allowed to reconstitute in certain slightly toxic solutions. Reagents which have been used to increase head frequency in the posterior levels of the first zooid are dilute KCN (Child, 1916), chloretone, chloroform, chloral hydrate, ether, ethyl alcohol (Buchanan, 1922), and caffeine (Hinrichs, 1924). Mechanical stimulation (Child, 1920) and abrupt change in temperature from 20° to 29° C. (Behre, 1918) have also been found to increase head frequency.

Child (1911*a*, 1912, 1916) has shown that the head frequency of pieces from anterior levels and of long pieces could be decreased by lowering the temperature or by allowing the pieces to reconstitute in dilute solutions of metabolic end-products, ethyl alcohol or KCN, for varying intervals of time. Behre (1918) found that animals, taken from a living temperature of 20° C., sectioned, and allowed to reconstitute in a temperature of 10° C., gave a decrease in head frequency. Buchanan (1922) found a slight decrease in the head frequency in

anterior pieces with short time exposure to anesthetics. Hinrichs (1924) found that anterior pieces showed a decreased head frequency with initial exposure of 24 hours to M/200 caffeine.

In general it would seem that those agents which increase head frequency do so either by inhibiting the effect from the posterior cut surface or by stimulating the head-forming cells at the anterior cut surface. A decrease in head frequency appears to be the result of an increased effect of the inhibiting factor or a decrease in the activity of the head-forming cells at the anterior cut surface.

Since the H-ion and CO₂ have been regarded as important factors in many biological processes, it was suggested that the reconstitution of *Euplanaria dorocephala* in different concentrations of H-ion and CO₂ might well be investigated. Experiments were also suggested which dealt with the possible effects of calcium in antagonizing the H-ion and with the experimental alteration of physiological condition of animals prior to section.

MATERIAL AND METHODS

The material used in this investigation was the common flatworm, *Euplanaria dorocephala*, which is found in great numbers in springs of the Valparaiso moraine to the south and west of Chicago. The chief localities for the collection of worms during this work were Cary, Illinois; Rockford, Illinois; and Valparaiso, Indiana. The worms were kept in large granite pans in the laboratory for several weeks before the experiments were performed. They were fed twice a week on beef liver, and the water was changed on alternate days.

Except when it was desired to bring out the effects of recent feeding, the worms were starved for approximately 2 weeks before they were used in the experiments. (Animals which have been recently fed usually show greater variability in head frequency, as well as a greater susceptibility.) Although 12-14, 14-16, and 15-17 mm. animals were used, by far the most experiments were performed with animals 14-16 mm. in length. Unless particular attention is called to this fact, it may be assumed that 14-16 mm. animals are being used.

Lots of 65 animals of the same length and breadth were chosen from the same stock for tests and controls. The test animals were al-

ways washed twice in the test solution before sectioning. Test animals were sectioned in their respective test solutions, while control animals were sectioned in well water.

In the head-frequency experiments the animals were cut in transverse sections in the manner shown in Figure 1. All the pieces were approximately one-eighth the length of the animal, exclusive of the head. Only the anterior zooids were used, and after a little practice it was possible to cut the pieces with a great deal of exactness. Of the 65 pieces from each region, 15 showing the greatest variations in length were discarded. This procedure considerably decreased any error related to faulty cutting.

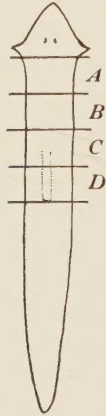


FIG. 1.—Levels A, B, C, and D used in all head-frequency experiments. Each section is approximately $1/8$ th the length of the animal, exclusive of the head. Head and region posterior to the mouth were discarded.

After section the pieces from the different levels were placed in 1-liter Erlenmeyer flasks which were filled with the test and control solutions. The flasks were tightly stoppered and left in the dark at room temperature until the pieces reconstituted. The test and control solutions were changed daily in a great many of the experiments, but in the majority of cases it seemed advisable not to stimulate the pieces by changing the solution. Other reasons for not

changing the solutions in which the worms are reconstituting will become clear as the individual experiments are described.

After 2 weeks the reconstituted animals were washed from the flasks and examined under a binocular microscope. They were given arbitrary values according to the type of head as follows: normal, 100; teratophthalmic, 80; teratomorphic, 60; anophthalmic, 40; acephalic, 20. These arbitrary values were multiplied by the frequency of the different head-forms, and the sum of all was divided by the total number of *living* animals to obtain the "head-frequency index." (This "head-frequency index" is identical with the "mean" described by Child and Watanabe, 1935.)

The test solutions were made up in 16-liter quantities as follows:

1. *Carbon dioxide solutions*.—Since the well water in the laboratory contains a large quantity of bicarbonates, it was decided to make use of the CO_2 that was liberated when HCl was added to the water. It was found that enough acid added to the well water to change the pH from 7.5 to 5.4 released approximately 60 cc. of CO_2 per liter. Changes in pH from 7.5 to 6.0, 7.5 to 6.6, and 7.5 to 7.0 released 45 cc., 18 cc., and 8 cc. of CO_2 per liter, respectively. The CO_2 liberated is, of course, not all in the gaseous form but is largely in such forms as H_2CO_3 , HCO_3^- , and =CO_3 . If the solution was allowed to stand in a container open to the air for a few hours, the CO_2 was expelled and the pH of the solution rose to 7.0 or above. As this method for obtaining CO_2 in solution was used, all tests involving the use of this reagent are recorded in terms of their pH values.

2. *Hydrogen ion solutions*.—It was highly desirable that the well water in the laboratory be used in making up the test solutions, as it contains the proper salts and was used for the controls. In order to be rid of the effect of CO_2 when testing the effect of pH, it was necessary to first free the water of bicarbonates. Smith and Clowes (1924) found that the bicarbonate could adequately be removed from sea water by adding enough concentrated HCl to break down all carbonates and bicarbonates and then removing the CO_2 by pumping air through the solution. Hyman (1925) used this method for removing the bicarbonates from well water.

The method used in these experiments for the removal of bicarbonates was similar to Hyman's method. Four and one-half cubic centimeters of concentrated HCl were added to 16 liters of well water, and air was bubbled through this solution for 24 hours. At the end of this time the CO_2 in the water was in equilibrium with the air and was found to be of no importance. The buffering capacity of this carbonate-free water almost entirely disappeared. The pH was adjusted to the desired values by adding minute quantities of $\text{M}/10$ HCl or $\text{M}/10$ NaOH . The exact pH value was determined by a quinhydrone apparatus supplemented with a series of La Motte color indicators.

THE EFFECT OF CARBON DIOXIDE ON HEAD FREQUENCY
AND VIABILITY

A number of experiments were performed in determining the exact concentrations of CO_2 the animals could tolerate. This procedure was necessary since a large number of experiments have shown that those concentrations which are most effective in altering head frequency are usually very near the lethal level. In fact, it has been found that solutions which cause cytolysis of a small percentage of the experimental pieces are usually the most effective.

Experiments in which intact animals 14–16 mm. in length were placed in CO_2 solutions ranging from 4.8 to 7.0 showed that cytolysis in the head regions often took place in concentrations below pH 5.2. Some worms even underwent complete disintegration at these concentrations. One-eighth pieces were quite susceptible to pH 5.2 and usually cytolized within a few hours. A concentration in which only a few pieces died was found at pH 5.4–5.6.

It was noted with much interest that animals which had lost only their heads in concentrations of pH 5.2 or below would reconstitute new normal heads in exactly the same solutions that had caused the cytolysis of the old head. It was also noted that those animals which had lost their heads, as well as animals whose heads had remained intact, often underwent fission in high concentrations of CO_2 . The percentage of fission was apparently closely related to the size and general physiological condition of the worms.

Another phenomenon, which was noted at this time and later made use of in experiments on "conditioning," was the acquiring of tolerance by intact animals to CO_2 . It was found that animals which had been kept in solutions of pH 5.4–5.6 for a few days could live, with no apparent injury, when transferred to solutions of pH 5.2. If kept in the latter solution for a few days, they could then be transferred to pH 5.0 without cytolysis resulting. In other words, animals living in certain concentrations of CO_2 for a time will acquire a tolerance for this reagent and, in doing so, will be able to survive solutions that are toxic to animals taken directly from well water, pH 7.5.

In the experiments on head frequency described in this section only worms which had been kept in freshly drawn well water until the time for experimentation were used. In the early experiments the solutions were changed daily; but, because of the large quantity of solution, as compared to the pieces, and the fact that there was little or no change in the solutions during the reconstititional period, this was given up as time-consuming. Other reasons for not changing the solutions were that the type of head appears to be determined by at least 2 or 3 days after section and the stimulation of pieces in

TABLE I
HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorotocephala* IN
CARBON DIOXIDE SOLUTIONS
(Head types and deaths given in percentages)

	WELL WATER (CONTROL)				CARBON DIOXIDE SOLUTION, pH 5.4-5.6			
	A	B	C	D	A	B	C	D
Normal.....	92.0	34.6	7.3	2.6	72.0	60.0	18.6	13.3
Teratophthalmic.....	8.0	40.0	5.3	0.6	5.3	14.0	20.0	8.0
Teratomorphic.....	2.0	4.6			1.3	0.6	2.6	4.6
Anophthalmic.....		10.0	21.3	10.3		4.6	15.3	6.0
Acephalic.....		10.6	60.6	86.6	1.3	10.0	29.3	22.6
Dead.....	2.0	0.6			20.0	10.0	14.6	45.3
Index.....	98.4	75.9	35.3	23.9	96.6	84.6	55.8	53.6

changing the solutions might present additional, not completely controllable factors.

In Table I are presented the head frequencies of three different experiments involving 150 test and 150 control worms. These experiments were performed on three different stocks and at three different times; but as each experiment shows the same relationship between tests and controls, these three different experiments are grouped together. It can readily be seen from this table that the head frequencies are distinctly higher in B, C, and D pieces which have reconstituted in the test solution. The relationship between tests and controls is shown graphically in Figure 2, in which the head frequency indices are plotted against body-level.

Another fact brought out in Table I is the different percentages of viability of pieces from different levels. The data at least indicate that *A* and *D* pieces are more susceptible to the toxic effect of

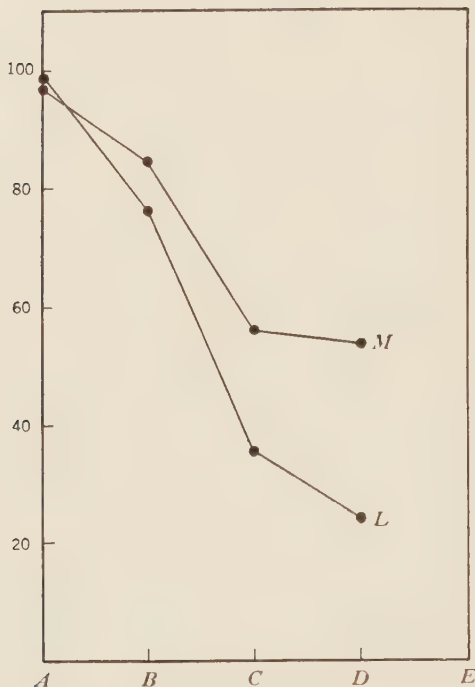


FIG. 2.—Head frequencies in CO₂ solution (*M*) and well water (*L*). Graph is plotted with the head-frequency indices as ordinates and body-levels as abscissae.

worms were of the same size and from the same stock. They were sectioned at the same time and serve as adequate controls for each other.

It will be noted that pieces which have reconstituted in pH 6.2 show a head frequency as high as, or possibly higher than, those which have reconstituted in pH 5.4; yet the number of deaths is practically zero. Solutions with a pH of 5.4 contain approximately 60 cc. of CO₂ per liter, while those with a pH of 6.2 contain approxi-

CO₂ than are pieces *B* and *C*. It may be that *A* pieces are more susceptible because of the normal higher activity of that particular level. The high susceptibility of *D* pieces may be attributed to the tremendous increase in respiratory metabolism and in susceptibility which these levels undergo following section. The presence of the pharynx in *D* pieces, may also have something to do with the greater susceptibility.

Experiments on head frequency were performed in which the concentrations of CO₂ were pH 5.4, 6.2, and 6.6. The head-frequency indices and the percentage of viability are shown in Table II. These

mately 38 cc. per liter. It appears from these data that pH values below 6.2 are more effective in increasing the number of deaths than the head frequency. At pH 6.6 the effect of CO₂ is still evident at *C* and *D* levels.

That this effect we have been describing is actually due to CO₂ rather than the H-ion is shown in Table III, in which the head-fre-

TABLE II
HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorocephala* IN
VARIOUS CONCENTRATIONS OF CARBON DIOXIDE

	HEAD-FREQUENCY INDEX				PERCENTAGE LIVING			
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
Well water (control).....	97.4	82.4	39.2	27.2	100.0	98.0	100.0	100.0
CO ₂ solution, pH 5.4.....	91.5	89.2	64.4	58.7	80.0	82.0	72.0	62.0
CO ₂ solution, pH 6.2.....	99.6	96.4	77.6	51.0	100.0	100.0	100.0	98.0
CO ₂ solution, pH 6.6.....	97.4	81.2	47.0	42.0	100.0	100.0	100.0	100.0

TABLE III
HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorocephala* IN
CERTAIN NON-EFFECTIVE HYDROGEN ION SOLUTIONS

	HEAD-FREQUENCY INDEX				PERCENTAGE LIVING			
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
Well water (control).....	98.8	81.6	41.0	31.8	100.0	100.0	72.0	100.0
Carbonate-free well water, pH 7.5.....	98.8	68.0	36.0	30.8	100.0	100.0	100.0	100.0
Carbonate-free well water, pH 5.6.....	99.0	78.0	34.6	27.4	100.0	100.0	100.0	100.0

quency indices of worms which have reconstituted in carbonate-free well water, pH 5.6 and 7.5, and in unaltered well water (control solutions), pH 7.5, are compared. According to Table III, the lots in carbonate-free well water at pH 7.5 and 5.6 show slightly lower head frequencies than the control, but the significance of the differences is doubtful. That the effect of H-ion alone at these concentrations is unimportant is fully confirmed by later experiments.

THE EFFECT OF THE HYDROGEN ION ON HEAD
FREQUENCY AND VIABILITY

All test animals dealt with in this section were allowed to reconstitute in carbonate-free well water which was prepared by the method described earlier in this paper.

Experiments on intact animals submitted to different concentrations of the H-ion show that this reagent has little effect between pH values of 7.2 and 4.2. Below 4.1 all of the animals usually cytolyzed. There was no evidence at this time of any acclimation to high concentrations, and no increase in fission rate was noted. If animals are left in pH 4.15 for several days, the pigmentation darkens and the tissues appear somewhat swollen. In alkaline solutions the animals died at a pH of approximately 10.0. In these solutions there was no evidence of any physiological changes before death.

A series of fifteen experiments was devised in which the head frequencies of the different levels of 50 animals were determined at every 0.4 pH value between pH 4.2 and 9.6. The solutions were changed daily throughout the entire reconstititional period. The worms used in these experiments were from the same lot and were sectioned at approximately the same time. From these fifteen experiments the only concentration that gave any evidence of inducing an increase in head frequency was pH 4.2. There was no effect whatever between pH 4.6 and 7.0. From 7.0 to 9.6 there appeared to be a slight decrease in the head frequencies of reconstituted *C* pieces.

As a pH of approximately 4.2 seemed to be quite important in the alteration of physiological conditions in reconstituting pieces, twelve different experiments, involving over 1,200 worms, were performed at pH ranges from 4.15 to 4.30. Each experiment consisted of 50 test and 50 control worms. These animals were from different localities and were collected at different times throughout the year. Although the head frequencies of the animals from different stocks did not always coincide, the head frequencies of the tests were always well above the head frequencies of the controls. The data from these different experiments were therefore grouped together in Table IV.

It will be noted from Table IV that the percentage of normal worms is much greater in the tests than in the controls, except in *A* pieces, and that the percentage of acephalic forms in the tests is

much smaller. A comparison of the head-frequency indices of worms which have reconstituted in critical concentrations of CO_2 (Table I) and of H-ion (Table IV) indicates that in these concentrations the H-ion may be more effective in increasing head frequency than CO_2 . It will also be noted that the death frequency in the tests at the different levels corresponds to the death frequency of pieces in high concentrations of CO_2 .

TABLE IV
HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorotocephala* IN
CARBONATE-FREE WELL WATER
(Head types and deaths given in percentages)

	WELL WATER (CONTROL)				CARBONATE-FREE WELL WATER, pH 4.15-4.3			
	A	B	C	D	A	B	C	D
Normal.....	91.1	43.7	13.0	8.3	76.6	51.2	35.8	35.7
Teratophthalmic.....	7.7	27.7	11.3	7.5	6.2	22.7	19.2	14.3
Teratomorphic.....	1.0	2.3	4.2	3.5	0.2	2.7	3.8	6.3
Anophthalmic.....	0.2	9.6	20.2	9.0	0.2	4.1	13.8	9.3
Acephalic.....		16.6	50.8	71.2		7.5	16.8	13.2
Dead.....			0.5	0.5	16.6	11.8	10.5	21.2
Index.....	97.9	74.4	43.0	34.3	98.1	84.0	69.6	72.7

Pieces that have reconstituted under conditions of high H-ion concentrations (pH 4.15) show morphological characteristics which are quite different from the controls. The pigmentation in the old tissues of the reconstituted test worms usually becomes very dark and diffuse. The new outgrowth may remain almost entirely unpigmented for a considerable length of time. Stunting of new tissue in both head and tail regions has been noted in such high concentrations of the H-ion.

CALCIUM ANTAGONISM TO THE HYDROGEN ION

Much work has been done in the past on the antagonism of ions. It has been found, in general, that the injurious effect of one ion may be obliterated by another ion even though the other ion alone may produce injuries. Divalent ions have been found to antagonize the actions of monovalent ions to a great extent. Calcium, particularly,

has been used in many fields of biological investigation to antagonize the injurious effects of sodium and potassium.

It has been established, in this work, that the H-ion in high concentrations has the property of altering reconstitucional development in *Euplanaria*. The question arises as to whether or not the effect of this ion can be antagonized by increasing the calcium content of the test solutions.

Preliminary experiments were carried out on intact worms in which from 0.1 to 1.0 gm. of calcium (CaCl_2 , anhydrous) were added per liter of test solution. The pH of the solutions was approximately 4.0. It was found that animals in solutions of the same pH would live a much longer time if the calcium concentration was increased. It apparently made little difference whether the calcium concentration was increased 0.1 or 1.0 gm. per liter.

A series of experiments was next conducted to determine if calcium could antagonize the effect of H-ion on head frequency. Six experiments were performed, which involved 900 worms from the same stock. Each experiment consisted of a control in well water, pH 7.5, a control in carbonate-free well water, pH 4.15, and a test in carbonate-free well water, pH 4.15, plus 0.5 gm. of CaCl_2 per liter. The results of these six experiments are tabulated in Table V. It will be noted from this table that worms allowed to reconstitute in carbonate-free well water at pH 4.15 have a characteristically higher head frequency than those which have reconstituted in well water, pH 7.5. This is not true in the case of pH 4.15, where the calcium concentration of the solution has been increased. Instead, the head frequency in these solutions approaches very closely the head frequency obtained in the well-water controls. In other words, the addition of calcium to these concentrations of the H-ion almost completely antagonized its effect on head frequency. Not only is this effect of the H-ion on head frequency decreased with calcium, but the death percentages of the pieces are likewise decreased under the same conditions.

In the light of the foregoing observations, a head-frequency experiment was performed, using as control solutions well water, pH 7.5, and carbonate-free well water, pH 4.1. The test solution was carbonate-free well water, pH 4.1, plus 0.5 gm. of CaCl_2 per liter.

The head frequencies and viability in this experiment are recorded in Table VI. It will be seen, in this case, that calcium antagonizes to a high degree the death-rate in high concentrations of H-ion but that the increase in head frequency is only partially antagonized.

TABLE V

ANTAGONIZING ACTION OF CALCIUM ON EFFECTIVE CONCENTRATIONS OF THE HYDROGEN ION

(Head types and deaths given in percentages)

	WELL WATER (CONTROL)				CARBONATE-FREE WELL WATER, pH 4.15				CARBONATE-FREE WELL WATER, pH 4.15, PLUS 0.5 Gm. CaCl ₂ (L)			
	A	B	C	D	A	B	C	D	A	B	C	D
Normal.....	92	21	3	4	78	40	21	23	93	36	4	4
Teratophthalmic.....	7	28	3	1	4	28	16	8	5	30	4	2
Teratomorphic.....	1	3	1	1	...	3	6	6	...	5	4	1
Anophthalmic.....	16	12	4	...	7	16	12	...	11	23	7	7
Acephalic.....	31	80	88	...	14	32	20	...	20	65	83	83
Dead.....	1	...	2	18	9	10	31	2	...	...	2	2
Index.....	98	59	27	25	99	76	55	61	99	70	32	27

TABLE VI

THE PREVENTION OF DEATH BY THE HYDROGEN ION WITH CALCIUM

	HEAD-FREQUENCY INDEX				PERCENTAGE LIVING			
	A	B	C	D	A	B	C	D
Well water (control).....	97.9	44.5	21.6	20.8	98.0	98.0	100.0	100.0
Carbonate-free well water, pH 4.1.....	...	...	90.0		0.0	0.0	8.0	0.0
Carbonate-free well water, pH 4.1, plus 0.5 gm. CaCl ₂ (L).....	99.0	81.7	40.0	42.9	74.0	94.0	92.0	96.0

It was of interest in considering the subject of ionic antagonism to test the viability and head frequency of highly susceptible animals (those fed 48-72 hours prior to section) in concentrations of H-ion, pH 4.15, with and without additional calcium. The results are shown in Table VII. In this case the death-rate is considerably antagonized,

while the head frequency is only partially antagonized by the action of calcium.

In order to test the effect of increased concentrations of calcium, aside from the antagonistic action on the H-ion, a series of experi-

TABLE VII
THE EFFECTS OF CALCIUM AND THE HYDROGEN ION ON
HIGHLY SUSCEPTIBLE ANIMALS

	HEAD-FREQUENCY INDEX				PERCENTAGE LIVING			
	A	B	C	D	A	B	C	D
Well water (control).....	100.0	65.3	34.1	28.3	90.0	84.0	88.0	96.0
Carbonate-free well water, pH 4.15.....	100.0	80.0	56.0	46.6	8.0	4.0	10.0	12.0
Carbonate-free well water, pH 4.15, plus 0.5 gm. CaCl ₂ (L).....	99.2	64.0	45.3	41.3	100.0	90.0	98.0	90.0

TABLE VIII
THE ACTION OF CALCIUM ALONE ON HEAD FREQUENCY AND
VIABILITY IN *Euplania dorotocephala*

	HEAD-FREQUENCY INDEX				PERCENTAGE LIVING			
	A	B	C	D	A	B	C	D
Well water, pH 7.5 (con- trol).....	99.2	64.4	22.5	20.0	100.0	100.0	96.0	96.0
Well water, pH 7.5, plus 0.5 gm. CaCl ₂ (L).....	100.0	71.2	24.7	21.2	100.0	100.0	100.0	100.0
Carbonate-free well water, pH 4.15.....	100.0	87.6	64.5	51.2	98.0	100.0	98.0	68.0
Carbonate-free well water, pH 4.15, plus 0.5 gm. CaCl ₂ (L).....	99.6	77.2	32.2	26.4	100.0	100.0	98.0	100.0

ments was performed in which the test solution was well water, pH 7.5, plus 0.5 gm. of CaCl₂ per liter. The control solutions were well water, pH 7.5, carbonate-free well water, pH 4.15, and carbonate-free well water, pH 4.15, plus 0.5 gm. of CaCl₂ per liter. The results, which are tabulated in Table VIII, indicate that calcium alone may slightly increase the head frequency. In this case it apparently has little effect on viability.

One head-frequency experiment was performed in which CaCl_2 (0.5 gm. per liter) was added to a test solution of CO_2 pH 5.4. This experiment was adequately controlled with solutions of CO_2 , pH 5.4, and well water, pH 7.5. It was found that calcium did not antagonize the effects of CO_2 either by decreasing the head frequency or the death percentages.

THE PHYSIOLOGICAL CONDITION OF *Euplanaria* IN NATURE

Throughout this investigation it has been found that control worms of the same size but from different stocks may yield very different head frequencies.

It has also been noted that worms from the same stock vary considerably from time to time as regards head frequency. Animals collected in the summer may show a different head frequency than those collected in the winter. It has been observed that worms in "poor" condition (i.e., many cytolyzing in the stock pans) usually show a higher head frequency than worms from the same stock under better conditions.

Worms of the same size and kept under the same laboratory conditions may behave differently if they are originally from different localities. In

Figure 3 are plotted the head-frequency indices of 150 control worms from each of three widely separated places.

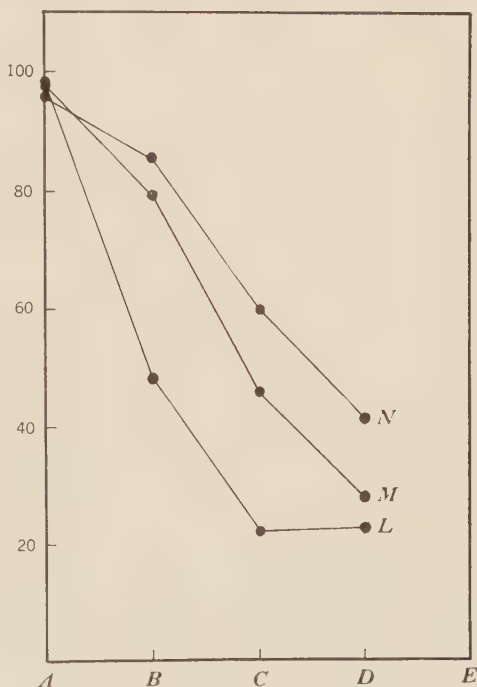


FIG. 3.—Head frequencies of different stocks of *Euplanaria dorotocephala* which have reconstituted in well water. Stocks were from Valparaiso, Indiana (L); Cary, Illinois (M); and Rockford, Illinois (N.)

It is not intended to imply by this graph that worms from different localities are persistently different. The head frequencies of worms (from any one locality) at different times in the year may vary as much as the three different head frequencies shown in the

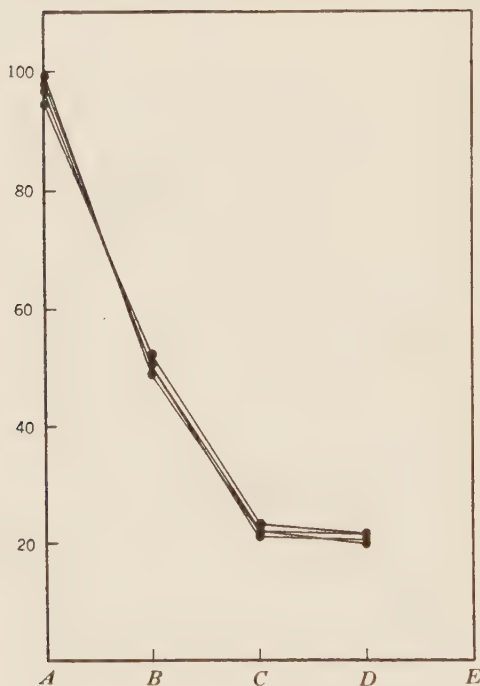


FIG. 4.—Head frequencies of four lots of worms from the same stock. They were identical in size and condition and were sectioned at approximately the same time. All were allowed to reconstitute in well water at the same temperature. (Worms were from Valparaiso, Indiana.)

graph. The only object of the graph is to show that the different lots of worms used in our experiments may be in different physiological conditions and that these conditions are expressed through the head frequencies. This difference in physiological condition must have been impressed upon the worms by their different environments prior to section.

But is there any pronounced difference in head frequency of different lots of worms of the same size, from the same stock, collected at the same time, sectioned at approximately the same time and kept under the same laboratory conditions? The answer to this question is given in Figure

4, in which the head-frequency indices of four control lots of 50 worms per lot, which met all the requirements, are plotted. It must be concluded from these data that like worms give like head frequencies and that all experiments on the alteration of head frequency have been adequately controlled.

It has been repeatedly observed that worms with controls of relatively high head frequency respond to the effects of such reagents as

H-ion and CO_2 with a greater increase in head frequency than stocks in which the controls show a low head frequency. Apparently animals in good condition (with low head frequency), although more susceptible than those in poor condition to directly lethal agents, are more capable of maintaining approximately normal internal conditions or of regulating these conditions when subjected to CO_2 or H-ion in the non-lethal concentrations used in these experiments. In other words, the animals in good condition tolerate these concentrations better, and so appear to possess a greater physiological stability within the experimental limits than those in poor condition.

THE EXPERIMENTAL ALTERATION OF PHYSIOLOGICAL
CONDITION IN *Euplanaria*

Since worms of the same size but from different environments yield different head frequencies under the same experimental conditions, a number of experiments were performed to determine whether the physiological conditions of animals could be altered in the laboratory in a short time by experimental means.

Three different methods for altering the physiological condition of the animals were tested. These were as follows:

1. Lots of 500-700 worms (14-16 mm.) were placed in a tightly stoppered 18-liter bottle in a solution of CO_2 , pH 5.6-5.7, for 4 days. This was followed by 6 days in CO_2 solution, pH 5.05-5.2. At the end of the conditioning period the worms were used for experimentation.

2. Lots of 500-700 worms (14-16 mm.) from the same stock as the worms in (1) were placed in carbonate-free well water, pH 4.15, in a tightly stoppered 18-liter bottle. The worms were left in this solution 9-10 days before sectioning for head frequency. The solution was changed daily.

3. Lots of worms of the same size and from the same stock as (1) and (2) were placed in well water, pH 7.5, in a tightly stoppered 18-liter bottle. The worms were kept under these conditions for 10 days prior to sectioning. The water was not changed from the beginning to the end of the conditioning period.

A control lot of 500-700 worms of the same size and from the same stock was maintained in an open pan, and the water changed daily.

Animals treated in the foregoing manner were taken directly from the experimental solutions, sectioned, and allowed to reconstitute in

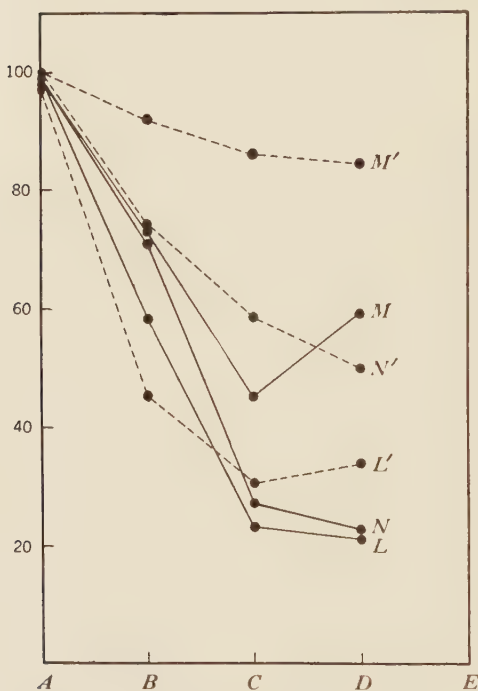


FIG. 5.—Head frequencies of CO_2 conditioned worms (broken lines) under different experimental and control conditions, as compared to non-conditioned worms. *L*, non-conditioned worms which have reconstituted in well water. *L'*, conditioned worms which have reconstituted in well water. *M*, non-conditioned worms which have reconstituted in carbonate-free well water, pH 4.15. *M'*, conditioned worms which have reconstituted in carbonate-free well water, pH 4.15. *N*, non-conditioned worms which have reconstituted in carbonate-free well water, pH 4.15, plus 0.5 gm. of CaCl_2 per liter. *N'*, conditioned worms which have reconstituted in carbonate-free well water, pH 4.15, plus 0.5 gm. of CaCl_2 per liter.

Carbon dioxide conditioned worms which had reconstituted in carbonate-free well water, pH 4.15, showed a considerably higher

well water, pH 7.5, in carbonate-free well water, pH 4.15, and in carbon-free well water, pH 4.15, plus 0.5 gm. of CaCl_2 per liter. The head frequencies were compared with non-treated control worms which had been submitted to the same test solutions during reconstitution.

1. *The effect of carbon dioxide on physiological condition.*—Animals conditioned with CO_2 gave by far the best results and showed clearly how the physiological gradients may be altered in intact animals before section.

Euplanaria conditioned to CO_2 and allowed to reconstitute in well water shows a higher head frequency in C and D pieces. In this experiment the conditioned B pieces may show a lower head frequency than the controls, as shown in Figure 5 (*L*, *L'*).

head frequency than non-conditioned worms in the same solution (Fig. 5 *M*, *M'*). In the non-conditioned worms the percentages of deaths were greater in *A* and *D* pieces. In the conditioned worms the death percentages (although as high) were not distributed in a regular fashion.

The non-conditioned worms reconstituting in pH 4.15 plus 0.5 gm. of CaCl_2 per liter (Fig. 5 *N*) give a head frequency almost as low as the non-conditioned worms reconstituting in well water (Fig. 5 *L*). In the conditioned worms (Fig. 5 *N'*), however, the antagonism of calcium to the H-ion decreases the head frequency below that of conditioned worms in H-ion alone (Fig. 5 *M'*), but it remains far above that of the non-conditioned worms (Fig. 5 *N*). Evidently the antagonism of calcium to the H-ion is not complete as regards head frequency in animals conditioned to CO_2 .

An interesting series of head-frequency experiments was performed on a separate lot of CO_2 conditioned worms in which pieces from the different levels were placed in CO_2 solution, pH 5.35, for given intervals of time after section. The pieces were then returned to well water, pH 7.5. In this series the reconstitution of the conditioned worms took place in (1) well water, pH 7.5, for the entire period, (2) CO_2 solution for the first 24 hours, (3) CO_2 solution for the first 48 hours, (4) CO_2 solution for the first 72 hours, and (5) CO_2 solution for the entire period. The head-frequency indices are presented in Table IX.

It will be noted from these data that all levels which have undergone CO_2 treatment, either before or after section, show an increased head frequency. It is of particular interest that conditioned pieces show a much higher head frequency when submitted to CO_2 for only 24-48 hours after section than with longer periods. (This effect may be because of a differential recovery or an activation of the head-forming cells that has been brought about by the change from an inhibiting solution to well water. If this is the case, we may assume that the exposure time was sufficient to inhibit the posterior-cut-surface factor.)

Animals conditioned in CO_2 , sectioned, and allowed to reconstitute for the entire period in CO_2 solution show a lower head frequency than would be expected from the results with non-condi-

tioned worms. This fact suggests that an acquired tolerance to CO_2 before section may result in less effect of CO_2 after section.

2. *The effect of the hydrogen ion on physiological condition.*—Animals conditioned with the H-ion and those not conditioned gave almost identical head frequencies when allowed to reconstitute in well water, pH 7.5. When reconstitution took place in carbonate-free well water, pH 4.15, the conditioned animals showed a distinctly higher head frequency in B, C, and D pieces than non-conditioned animals in the same solution.

TABLE IX

HEAD FREQUENCY AND VIABILITY OF ANIMALS CONDITIONED TO CARBON DIOXIDE AND ALLOWED TO RECONSTITUTE FOR VARIOUS INTERVALS OF TIME IN THE SAME SOLUTION

	HEAD-FREQUENCY INDEX				PERCENTAGE LIVING			
	A	B	C	D	A	B	C	D
Well water (control)	97.9	44.5	21.6	20.8	98.0	98.0	100.0	100.0
T-1, well water, pH 7.5	100.0	58.8	41.2	30.4	100.0	100.0	100.0	100.0
T-2, CO_2 solution, pH 5.35, 24 hours only	100.0	88.6	70.4	70.8	90.0	88.0	92.0	74.0
T-3, CO_2 solution, pH 5.35, 48 hours only	100.0	90.9	73.2	79.4	96.0	100.0	82.0	68.0
T-4, CO_2 solution, pH 5.35, 72 hours only	100.0	86.5	65.1	59.6	100.0	98.0	94.0	92.0
T-5, CO_2 solution, pH 5.35, entire period	99.6	84.9	65.8	58.8	98.0	82.0	90.0	90.0

Worms conditioned with the H-ion and allowed to reconstitute in pH 4.15 plus 0.5 gm. of CaCl_2 per liter gave a very interesting result. Instead of calcium causing a decrease in head frequency, as it did in the case of non-conditioned worms, it actually caused an *increase* in head frequency at all four levels. In other words, in the case of H-ion conditioned worms, the head frequency in high concentrations of the H-ion with calcium was higher than in the same concentrations of H-ion without calcium.

Death of pieces in pH 4.15 was almost entirely prevented by conditioning the worms to H-ion prior to section.³

³ Previous experiments with whole animals had given no visible evidence of an acquirement of tolerance to high concentrations of the H-ion. These experiments indicate,

3. *The effect of standing well water on physiological condition.*—Experiments comparable to those performed on worms conditioned to the H-ion and CO₂ were performed on worms conditioned to standing well water. It may be briefly stated that the head frequencies of worms conditioned in this manner were not substantially different from those of non-conditioned worms.

THE EFFECT OF THE HYDROGEN ION ON DIFFERENTIALS
IN RATE OF EYESPOT FORMATION

It has been shown quite definitely throughout this investigation that there actually is a head-frequency gradient in *Euplanaria* and that this gradient can be altered by means of different reagents in the external medium. In this section we are not concerned with the head frequency except to point out the parallelism between it and another gradient, namely, the gradient in rate of eyespot formation. In speaking of the different gradients it is not meant to imply that there are different physiological gradients but that the different phenomena observed are different manifestations of the same axial differential.

Watanabe (1935a) has shown a gradient in rate of eyespot formation in this species when the different levels were allowed to reconstitute in well water. The object of the investigation here described is to determine whether external reagents can alter the eyespot gradient in a manner analogous to the alteration of the head-frequency gradient.

Animals in lots of 50 were sectioned in the manner shown in Figure 6. The section designated *X* comprises the anterior 5/16ths of the animal (exclusive of the head), while *Y* is the 5/16ths of the animal immediately posterior to *X*. Controls reconstituted in well water, pH 7.5, while the tests were submitted to carbonate-free well water, pH 4.20–4.15, for the reconstitutive period. The pieces were first examined at 36 hours after section. They were then examined every 12 hours until all showed definite eyespots. All examinations were made with the aid of a compound microscope. All the pieces of the size here described reconstituted into animals with normal heads.

however, that there actually is some readjustment of the intact animals to pH. It appears from these data that the acquirement of tolerance for the H-ion is considerably less than for CO₂.

It is sometimes quite difficult, especially in *A* pieces, to tell with certainty the exact time of the first appearance of the eyespots. This is primarily due to the accumulation of pigment at the site of injury, which renders the minute eyespot invisible. Pieces recorded as having eyes at any particular time always showed the eyespots distinctly.

By recording the number of animals that had developed eyes during each 12-hour interval, it was possible to get a mean rate of eyespot formation with a standard error. From data obtained from the different levels under control and experimental conditions it was possible to demonstrate any differential in rate of eyespot formation, as well as any alterations which may have been caused by external factors.



FIG. 6.—Sections *X* and *Y* (approximately 5/16ths of animal, exclusive of head) used in experiments on gradient in rate of eyespot formation.

The data for the rate of eyespot formation in pieces *X* and *Y*, as well as the head frequencies of 1/8th pieces (*A*, *B*, *C*, *D*, Fig. 1), from the same stocks of animals sectioned at the same time and allowed to reconstitute under the same test and control conditions are presented in Table X. In the first section of Table X are given the head-frequency indices. In the second section are given the rates of eyespot appearance in pieces *X* and *Y*. In the third section are given the differences in rate of eyespot appearance between (1) *X* and *Y* levels of both tests and controls ($Y - X$), (2) between *X* levels of tests and *X* levels of controls ($X_{II} - X_I$), (3) between *Y* levels of tests and *Y* levels of controls ($Y_{II} - Y_I$), and (4) between the differences found in (2) and (3) [$(X_{II} - X_I) - (Y_{II} - Y_I)$].

The first experiment on rate of eyespot formation was performed in midwinter at a temperature of approximately 20° C. It will be noted (Lot 1, Table X) that the control worms showed a relatively high head frequency, while the differential in rate of eyespot formation between levels *X* and *Y* was quite small (3.6 ± 0.95 hours). In the test worms the head-frequency gradient had almost entirely dis-

appeared, and the eyespot gradient was of no significance (0.7 ± 1.43 hours). In comparing the rate of eyespot formation in the tests and controls at the same levels, it was found that the rate of development was definitely inhibited in high concentrations of the H-ion (10 ± 1.39 hours for X levels and 6.3 ± 1.14 hours for Y levels). It appears from these data that the rate of eyespot formation was inhibited to a

TABLE X

THE GRADIENT IN RATE OF EYESPOT FORMATION IN *Euplanaria dorotocephala* WITH ITS ALTERATION BY USE OF THE HYDROGEN ION
(Rate of eyespot appearance given in hours)

	HEAD-FREQUENCY INDEX				RATES OF EYESPOT APPEARANCE		DIFFERENCES IN RATE OF EYESPOT APPEARANCE			
	A	B	C	D	X	Y	$Y-X$	$X_{II}-X_I$	$Y_{II}-Y_I$	$(X_{II}-X_I)-(Y_{II}-Y_I)$
Lot 1	I. Well water (control)				73.9 ± 0.82	77.5 ± 0.49	3.6 ± 0.95	10.6 ± 1.39	6.3 ± 1.14	4.3 ± 1.71
	II. Carbonate-free well water, pH 4.2				84.5 ± 0.98	83.8 ± 1.03	-0.7 ± 1.43			
Lot 2	I. Well water (control)				50.4 ± 0.86	64.1 ± 1.05	13.7 ± 1.35	9.4 ± 1.54	3.8 ± 1.56	5.6 ± 2.2
	II. Carbonate-free well water, pH 4.15				59.8 ± 1.28	67.9 ± 1.16	8.1 ± 1.73			

greater extent in X than in Y pieces (4.3 ± 1.71 hours). It seems to be quite clear that in the test solutions the physiological gradient may be flattened out or obliterated through differential inhibition.

The second experiment on rate of eyespot formation (Lot 2, Table X) was performed in the summer at a temperature of approximately 32°C . It will be noted that the control worms showed a very low head frequency, while the differential in rate of eyespot formation in pieces X and Y of similar worms was quite marked (13.7 ± 1.35 hours). The test worms (those which reconstituted in carbonate-free well water, pH 4.15) showed a higher head frequency in pieces B , C , and D and less differential in rate of eyespot formation

at levels *X* and *Y* (8.1 ± 1.73 hours). As in the case of the foregoing experiment, both levels are retarded in rate of eyespot formation (9.4 ± 1.54 hours for level *X* and 3.8 ± 1.56 hours for level *Y*). Here, again, level *X* is retarded more than level *Y* (5.6 ± 2.2 hours). In this later experiment the differential was so great between the different levels that it was only partially destroyed by the H-ion.

DISCUSSION

It has been pointed out repeatedly during this work that the inhibition of heads in short transverse pieces of *Euplanaria* is the result of some factor or factors arising from the posterior cut surface. This inhibiting influence may be present for several days and, as stated by Child and Watanabe (1935) and Watanabe (1935*b*), it exerts its effect by preventing in some way the dedifferentiation and activation of the head-forming cells in the anterior region of the piece. These authors have given evidence indicating that this inhibitory effect of posterior cut surface is transmitted forward from its point of origin chiefly by way of the ventral nerve cords.

It has also been found (Watanabe, 1935*a*) that a gradient in rate of head growth (eyespot formation) exists along the axis. This gradient of eyespot formation is apparently independent of any posterior effect, since it appears in long pieces in which head development is not inhibited. Those regions which show the slowest rate in eyespot formation are likewise the regions which give the largest number of inhibited head-forms with short transverse pieces.

It has been suggested (Child and Watanabe, 1935) that the inhibitory effect arising from the posterior cut surface may not come into play in transverse pieces from the more anterior levels because of a greater activity of the head-forming cells in these regions. It was also suggested that a decrement in the transmission of the inhibiting factor may be greater in the anterior levels and less in the posterior levels of the first zoid.

When anterior and posterior section are made at the same time in 1/8th *D* pieces, head frequency is low and acephalic forms predominate; while a delay in the posterior cut for 6–8 hours will result in the reconstitution of animals with 100 per cent heads, and with longer delay 100 per cent normal heads. From this it seems to be evident

that a short delay in bringing into action by posterior section the factor inhibiting head development is enough to permit at least a beginning in dedifferentiation and activation of the head-forming cells. With this advantage the anterior end is able to carry through the reconstitution of a normal head in spite of any inhibiting effect which later may be imposed from the posterior cut surface.

It seems quite clear that the increase in head frequency found in the experiments with CO_2 and H-ion is brought about through some kind of inhibition of the factor inhibiting head development. These agents may act directly in decreasing activity at the posterior cut surface which inhibits head formation, or they may act by penetrating and blocking any excitation passing forward from the posterior point of origin. It has been found in the experiments on rate of eyespot formation that carbonate-free well water with low pH (Table X) retards, to a certain extent, the whole reconstitutive process, while it increases head frequency. (Child, 1916, observed this same phenomenon with dilute solutions of KCN.) Because of this fact, it seems more logical to assume that the inhibition of the factor inhibiting head development is one aspect of a general inhibiting action which is more effective in decreasing or eliminating that factor, perhaps by block, than in inhibiting the head-forming cells.

The effects of carbon dioxide.—It has been seen from the experiments on head frequency that CO_2 and the H-ion behave quite differently in the alteration of head frequency even though the end results may be of like nature. It appears as if CO_2 penetrates and produces its inhibiting effects quite independently of external pH. It has been found (Osterhout and Dorcas, 1925) that in *Valonia* little or no CO_2 enters normal cells except in the form of undissociated molecules. Since HCl ionizes completely in the concentrations used and H_2CO_3 is only partly ionized, it seems probable that at least some of the differences in the effects observed are related to the differences in penetration of CO_2 and the H-ion. Hyman (1925) has found that CO_2 depresses considerably the oxygen consumption in *Euplanaria*. Haywood (1927), in describing CO_2 as a narcotic agent, has shown that high concentrations almost completely suppress cleavage in the eggs of *Arbacia*. Jacobs (1912, 1920a, 1920b, 1922) has shown that the action of CO_2 is apparently independent of the

H-ion and that H_2CO_3 differs from the strong acids in its ability to penetrate living cells. Its action has been described by this author as being more or less specific. He found that CO_2 produces first a liquefaction, followed by coagulation of the protoplasm of *Spirogyra*. Fox (1932, 1933a, 1933b) goes into the subject of CO_2 effects, particularly on the streaming of protoplasm, and attributes the phenomena observed to CO_2 narcosis rather than a H-ion effect. In considering the subject of CO_2 versus H-ion, Heilbrunn (1928, p. 188) points out that CO_2 in solution is more or less anesthetic in action, has a lower surface tension than the pure solvent, and may act as a fat solvent in decreasing protoplasmic viscosity.

Rustia (1925) found that HCl added to well water increased the number of bipolar forms arising from short transverse pieces of *Euplanaria*. He also observed an increase in head frequency with this treatment. As he did not remove the bicarbonates from the well water, it is probable that his results are due to CO_2 rather than the H-ion.

In the experiments with CO_2 on head frequency described in this paper, it has been shown that CO_2 is effective over a wide range of concentrations and that the effect does not depend on the external H-ion. It has also been shown that worms submitted gradually to high concentrations of CO_2 will acquire a tolerance to solutions toxic to worms not so treated. It appears, in view of the previous work done on CO_2 , that all processes pertaining to growth are retarded but that the inhibitory factor arising from the posterior cut surface is affected to a greater extent than the head-forming cells. With this condition existing, it is clearly seen how short transverse pieces of *Euplanaria* which normally give a low head frequency may give increased head frequency in CO_2 .

The effects of hydrogen ion.—In our experiments with carbonate-free water it has been possible to demonstrate the actual effect of H-ion on reconstitution in *Euplanaria*. It has been shown, in the experiments on rate of eyespot formation, that the H-ion in the proper concentration retards, to a measurable extent, reconstitucional development. In concentrations below a critical value (pH 4.3) there is apparently little or no visible effect of the H-ion, while in higher concentrations (pH 4.1 or below) death results. This effect of

the H-ion is doubtless due to the impermeability of living membranes to low concentrations of the ions of strong acids.

Osterhout (1914), in measuring the permeability of tissues of *Laminaria saccharina* by means of their electrical resistance, found that HCl produced a rapid decrease of permeability, which was shortly followed by a rapid increase, which continued until the death-point was reached. In the light of these and other experiments it appears probable that the H-ion does not enter the planarian tissue below a certain concentration (pH 4.3) because of permeability factors. Through a short range of higher concentrations the permeability of the tissues for the H-ion increases, and this results in an increase in the internal acidity. This fall of internal pH may result first in a decreased viscosity of the protoplasm (Heilbrunn, 1928). With a further decrease in pH, coagulation of the protoplasm occurs. It seems extremely likely that metabolic processes are affected by changes in internal pH.⁴

Jewell (1920), in her work on environment and regeneration, found that certain concentrations of HCl in distilled water retarded CO₂ production and regenerative growth in tadpoles. In Table 6 of her paper she records a pH of 5.4 as being inhibitory to these processes. She reports a concentration of N/0.00025 HCl as having a pH of 5.4; N/0.0005 HCl, a pH of 3.8; and N/0.00075 HCl, a pH of 3.2. From these figures it appears that some other factor besides pH is concerned, especially in solutions of pH 5.4. Hyman (1925) found that strong acids in carbonate-free well water had absolutely no effect on rate of oxygen consumption in planarians between pH 7.5 and 5.0. A slight depression (about 15 per cent) was found at pH 4.0. Heilbrunn (1928) suggests that the H-ion may liberate CO₂ from protoplasmic buffers and therefore act in much the same way as the direct penetration of CO₂. This suggestion is invalidated, at least in part, by Barth (1929), who shows that a number of strong and weak acids will produce coagulation in *Arbacia* eggs at the same external pH if the permeability factor is removed with NaCl. (At the same pH the different acids would be in different concentrations. The

⁴ It is apparent that CO₂ also alters the internal pH, and indeed it seems probable that these pH changes may be equally as important as the narcotic action of CO₂ in altering reconstititional development.

amount of CO_2 liberated depends on the concentration of the acid rather than on the pH.)

In the case of the H-ion it is apparent that the head frequency in *Euplanaria* is increased by a greater inhibition of the effect of the posterior cut than of the head-forming cells. It is also of much interest that the anterior levels of the first zooid are retarded in rate of growth to a greater extent than the more posterior levels of the same zooid. As the anterior levels are originally more active, this greater retardation is a good example of differential inhibition, a phenomenon that has been noted so often in this laboratory.

Calcium antagonism to the hydrogen ion.—It has been shown in these experiments that certain effective concentrations of the H-ion can be almost completely antagonized, as regards head frequency, by increasing the calcium concentration of the solution. It is known that calcium is used by living cells in the formation of such structures as the plasma membrane. Salts like sodium and potassium increase the permeability and break down cell membranes. The antagonistic action of calcium on sodium and potassium is too well known to need mentioning. It seems in these experiments that calcium antagonizes the activity of the H-ion by preventing the increase in permeability of the cell membranes which accompanies high concentrations of the H-ion. It is also possible that the antagonistic action of calcium on H-ion may be accomplished to some extent by the different actions of these agents on the protoplasmic colloids.

The experimental alteration of physiological condition.—It has been seen throughout these experiments that different lots of animals of the same size often have quite different head frequencies under the same experimental conditions. It appears probable that animals giving a high head frequency at the more posterior levels of the anterior zooid have less axial differential than those giving low head frequencies. However, since head frequency depends on two antagonistic factors (the capacity of the cells concerned in head formation to react to conditions resulting from anterior section, and the factor inhibiting activation and dedifferentiation of these cells which originates at the posterior cut surface), it is evident that the relation between the head-frequency gradient and the general axial differential is not simple and direct. The condition of the head-forming cells and

the effectiveness of the inhibiting factor may vary and can be altered experimentally in either direction, and head frequency in any given case depends on the relation between them. The differences in time of eyespot appearance at different body-levels (Table X) indicate, first, a graded difference in condition of the head-forming cells at different levels, which is apparently a direct expression of the general axial differential, and, second, variation in the amount of this difference in different stocks and under different experimental conditions, which suggests differences in steepness of the general gradient. It is not yet known, however, whether the factor inhibiting head development shows similar graded differences. In the light of the facts at present available, the head-frequency gradient appears to be an indirect and complex expression of the axial gradient involving two opposing and variable factors.

The object of the experiments on conditioning to CO₂ was to alter, if possible, the relation between these two factors, and so to alter head frequency. As is evident from Table IX and Figure 5, the head frequencies of conditioned animals are in all cases higher at the more posterior levels than in the non-conditioned; but there is no significant alteration in head frequency at anterior levels. It seems probable in the light of these results that the chief—perhaps the only—effect of the conditioning is the inhibition of the factor inhibiting head development at the more posterior levels. Since this inhibiting factor is apparently chiefly or wholly nervous in character, the conditioning in this case probably consists chiefly in the narcotic action of CO₂. The conditioned animals behave as if partially narcotized. They show decreased motor co-ordination and frequently undergo fission, as they do in other narcotics. Certainly the conditioning to CO₂ has little or no effect on the head-forming cells, even at the most susceptible anterior levels, for there is no decrease in head frequency there; and, since the inhibiting factor is not effective at anterior levels in 1/8th pieces, there is no increase. Obviously, head frequency can be altered at the more posterior levels by conditioning to CO₂ before sectioning, but the data indicate that the effect is chiefly on the nervous system rather than on the general axial gradient. The very great increase in head frequency in animals conditioned to CO₂ and subjected to H-ion after section (Fig. 5 *M'*) evidently results from the

combined effect of CO_2 in the protoplasm and the H-ion administered. The combined effect of the two agents may provide a more effective block against the inhibitory factor, but it does not necessarily follow that their effects are directly additive physiologically. If this were the case, an increase in percentage of deaths over the non-conditioned lot would be expected, since both CO_2 and H-ion are near the lethal concentration; but such increase does not appear.

The data on time of appearance of eyespots (Table X) suggests that some degree of leveling out of the general axial gradient does occur in the H-ion concentration used. The increase in time in H-ion is greater at the anterior level (X) than at the posterior level (Y) in both lots; and the difference in time between X and Y is less in the experimental than in the control lots. The pieces used in these experiments are long enough to give 100 per cent normal heads. Consequently, the times of appearance of eyespots are regarded as indicating differences in condition of the cells concerned in head formation at different levels.

SUMMARY

Euplanaria dorotocephala, 14-16 mm. in length, were cut into short transverse pieces (approximately $1/8$ th the length of the animal, exclusive of the head). Only the first four pieces (A, B, C, D), which make up most of the anterior zooid, were used. These pieces were caused to reconstitute under various experimental conditions. Upon reconstitution the head frequencies and the number dead at each level were recorded. All experimental lots were controlled with similar lots in well water.

1. Lots of reconstituted worms from animals taken directly from well water, sectioned, and permitted to reconstitute in CO_2 solution (pH 5.4-5.6) show an increased head frequency in levels B, C , and D . The death frequencies were greater in pieces A and D .

2. Intact animals submitted to high concentrations of CO_2 for a few days will become acclimated to CO_2 and be able to withstand considerably higher concentrations or concentrations which are lethal to animals that have not been previously treated with this reagent.

3. The effects of CO_2 are not due to the external pH, as experiments with carbonate-free well water show that the H-ion has little

or no effect on reconstitution in *Euplanaria* at the pH values of the CO₂ solution.

4. Head-frequency experiments in which pieces reconstituted in carbonate-free well water, pH 4.15-4.30, indicate that this concentration of the H-ion induces an increase in head frequency at levels *B*, *C*, and *D*. As in the case of CO₂, the death frequencies are greater in *A* and *D* pieces.

5. When CaCl₂ (0.5 gm. per liter) is added to carbonate-free well water, pH 4.15, the effect of the H-ion in increasing head frequency is almost completely antagonized. Animals are also protected from the lethal effects of the H-ion by the addition of calcium to the test solution.

6. Stocks of worms from different localities and different lots of worms from the same locality (collected at different times) may yield very different head frequencies under the same experimental conditions. Worms from the same locality and collected at the same time may vary as regards head frequency, depending on the physiological condition of the stock at the time of section and upon the length of time the worms have been kept in the laboratory.

7. Worms have been experimentally conditioned by submitting them to high concentrations of CO₂ and H-ion for several days before section. These conditioned worms react quite differently, as regards head frequency, than do non-conditioned worms from the same stock. The chief difference is an increased head frequency in the conditioned worms. Carbon dioxide is a much more effective agent for conditioning than H-ion.

8. In experiments on rate of eyespot formation at different body-levels in *Euplanaria* it was found that 5/16-*X* pieces (anterior 5/16ths of animal exclusive of the head) developed eyespots before 5/16-*Y* pieces (middle 5/16ths of animal). In carbonate-free well water (pH 4.15-4.20) the time of eyespot formation was longer at both levels, but *X* was retarded to a greater extent than *Y*.

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